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A Prothrombinase-based Assay for Detection of Resistance to Activated Protein C

Gerry A. F. Nicolaes[1], M. Christella L. G. D. Thomassen[1], Rene van Oerle[2], Karly Hamulyak[2], H. Coenraad Hemker[1], Guido Tans[1], Jan Rosing[1]

From the [1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM) and the [2]Department of Hematology, University Hospital, University of Limburg, Maastricht, The Netherlands

Summary

In this paper we present a new method for the detection of resistance to activated protein C (APC) that is based on direct measurement of the effect of APC on the cofactor activity of plasma factor Va. The factor V present in a diluted plasma sample was activated with thrombin and its sensitivity towards APC was subsequently determined by incubation with phospholipids and APC. The loss of factor Va cofactor activity was quantified in a prothrombinase system containing purified prothrombin, factor Xa and phospholipid vesicles and using a chromogenic assay for quantitation of thrombin formation. The reaction conditions were optimized in order to distinguish normal, heterozygous and homozygous APC-resistant plasmas. Maximal differences in the response of these plasmas towards APC were observed when factor Va was inactivated by APC in the absence of protein S and when the cofactor activity of factor Va was determined at a low factor Xa concentration (0.3 nM).

Addition of 0.2 nM APC and 20 μM phospholipid vesicles to a 1000-fold diluted sample of thrombin-activated normal plasma resulted in loss of more than 85% of the cofactor activity factor Va within 6 min. Under the same conditions, APC inactivated ~60% and ~20% of the factor Va present in plasma samples from APC-resistant individuals that were heterozygous or homozygous for the mutation Arg^{506}→Gln in factor V, respectively. Discrimination between the plasma samples from normal and heterozygous and homozygous APC-resistant individuals was facilitated by introduction of the so-called APC-sensitivity ratio (APC-sr). The APC-sr was defined as the ratio of the factor Va cofactor activities determined in thrombin-activated plasma samples after 6 min incubation with or without 0.2 nM APC and was multiplied by 100 to obtain integers (APC-sr = {factor Va_{+APC}/factor Va_{-APC}} × 100). Clear differences were observed between the APC-sr of plasmas from normal healthy volunteers (APC-sr: 8-20, n = 33) and from individuals that were heterozygous (APC-sr: 35-50, n = 17) or homozygous APC resistant (APC-sr: 82-88, n = 7). There was no mutual overlap between the APC-sr of normal plasmas and plasmas from heterozygous or homozygous APC resistant individuals ($p < 0.0001$). In all cases our test gave the same result as the DNA-based assay. Since the test is performed on a highly diluted plasma sample there is no interference by conditions that affect APC resistance tests that are based on clotting time determinations (e.g. coagulation factor deficiencies, oral anticoagulation, heparin treatment, the presence of lupus anticoagulants, pregnancy or the use of oral contraceptives). Furthermore, we show that part of the factor Va assay can be performed on an autoanalyzer which increases the number of plasma samples that can be handled simultaneously.

Correspondence to: Dr. J. Rosing, Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands – FAX Number: +31 43 3670 988

Introduction

In 1993, Dahlbäck et al. (1) described a hereditary defect in the anticoagulant response to activated protein C (APC) that is associated with an increased risk for venous thrombosis. This abnormality, called APC resistance, was subsequently identified as the most common defect in thromboembolic patients (2-6) and was found in 20-50% of patients with venous thrombosis. APC resistance is associated with familial thrombophilia and is inherited as an autosomal dominant trait. The molecular defect responsible for APC resistance is a single point mutation in the gene encoding for coagulation factor V. This mutation results in the substitution of Arg^{506} by Gln in the heavy chain domain of factor V(a) (7-11). Since Arg^{506} constitutes one of three APC cleavage sites in factor Va (12), the mutated factor V(a), also described as factor V^{Leiden} or factor V^{R506Q}, is much less effectively inactivated by APC than normal factor Va (13, 14). Since APC-catalyzed inactivation of factor Va is a crucial step in the down-regulation of thrombin formation this likely explains the increased risk for venous thromboembolism in individuals with APC-resistant plasma.

The requirement for reliable detection of APC resistance became important when it was discovered that at least 2 to 4% of the Caucasian population is APC-resistant (2, 3, 6, 7). Several methods for the diagnosis of APC resistance have been described so far (15). Most of these methods are based on sequence analysis of genomic DNA (7, 16) or on measurement of the effect of APC on the activated partial thromboplastin time (APTT) (1, 17, 18) or on thrombin generation in plasma (19). APTT-based APC-resistance assays appear to be less reliable in the case of other coagulation disorders (17, 20-22), anticoagulant therapy (2, 4, 6, 23), pregnancy (21) or the use of oral contraceptives (24, 25).

In this paper, we report a different method for the identification of APC-resistant plasmas. The method is based on direct measurement of the effect of APC on the activity of factor Va in diluted thrombin-activated plasma samples and subsequent determination of factor Va cofactor activity in prothrombin activation.

Materials and Methods

Materials and Buffers

The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. The chromogenic substrates D-Phe-

(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Recombinant human APC (rAPC) was a kind gift of Immuno AG, Vienna, Austria. Human protein S was purchased from Enzyme Research Laboratories, Swansea, United Kingdom. Human prothrombin and human factor X were purified as described by DiScipio et al. (26). Human factor X was converted into factor Xa by incubation with the purified factor X activator from Russel's viper venom and factor Xa was isolated from the activation mixture by chromatography on soybean trypsin inhibitor-Sepharose (27).

All proteins used in the APC resistance test were diluted in buffer I (25 mM Hepes [pH 7.5], 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg/ml BSA).

Phospholipid Vesicle Preparations

Small unilamellar phospholipid vesicles composed of a mixture of DOPS/DOPC (10/90, M/M) were prepared as described earlier (28). Phospholipid concentrations were determined by phosphate analysis (29).

Protein Concentrations

APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al. (30). Thrombin concentrations were determined with the chromogenic substrate S2238 (31). Protein S concentrations were calculated from the A_{280} using an $A^{1\%}_{280}$ of 9.5 and Mr = 70,000 for protein S (32). Factor V and factor Va concentrations were determined by measuring their activity in prothrombin activation (14, 33) under conditions described in the legends to the figures.

Collection and Handling of Plasma Samples

Nine parts of blood from normal healthy volunteers or from individuals (both men and women) that were shown to be heterozygous or homozygous for the Arg^{506}→Gln mutation in factor V by DNA analysis (34) were collected in one part of 0.13 M trisodium citrate (pH 7.8). The blood was centrifuged twice for 15 minutes at 3,000 × g at room temperature. The platelet poor plasma thus obtained was stored at -80° C. The healthy volunteers had a normal APTT and prothrombin time, a normal response to APC and DNA analysis showed that they were not carrying the Arg^{506}→Gln mutation. A normal plasma pool was obtained by mixing the plasma of 84 different healthy volunteers (both men and women). Coagulation factor concentrations (including protein S and C) of all volunteers were within the normal range.

Chromogenic APC-resistance Test

The procedure that we have developed basically consists of three steps that are performed at 37° C: 1) activation of plasma factor V with thrombin, 2) incubation of factor Va with or without APC and 3) quantification of factor Va cofactor activity.

Step 1: To two tubes (samples A and B) with 215 µl of 1/1000 dilutions of the same plasma sample in buffer I, 5 µl of a mixture of 175 nM thrombin and 1 mM phospholipid vesicles (10/90 M/M DOPS/DOPC) were added to activate the factor V present in the plasma sample.

Step 2: After 10 min 10 µl buffer I was added to sample A and 10 µl purified rAPC (4.6 nM) was added to sample B and the incubation at 37° C was continued.

Step 3: After 6 min incubation with APC, 10 µl prothrombin (25 µM) and 10 µl factor Xa (7.5 nM) were added to the samples A and B in order to determine the factor Va cofactor activity present. After 1 min, the amount of thrombin generated in the reaction aliquots was quantitated by transferring a 10 µl aliquot into a disposable cuvette with 990 µl of a buffer containing 235 µM S2238 in 50 mM Tris (pH 7.5 at 37° C), 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA. The factor Va concentration in the plasma sample was calculated from the thrombin formed using a calibration curve made with known amounts of purified factor Va (34).

The final concentrations of essential reaction components in the different steps were: step 1) 1/1000 dilution of plasma sample, 4 nM thrombin and 22.7 µM DOPS/DOPC (10/90, M/M) vesicles; step 2), 0.2 nM APC and 21.7 µM DOPS/DOPC (10/90) vesicles; step 3) 0.25 µM prothrombin, 0.3 nM factor Xa and 20 µM DOPS/DOPC (10/90) vesicles.

The factor Va assay procedure (step 3) is essentially the same as reported earlier (33). However, in this particular application factor Va was assayed at 0.3 nM factor Xa since at this factor Xa concentration the residual cofactor activity of the reaction intermediate cleaved at Arg^{506} is negligible compared to native factor Va (14). This increased the difference in response between normal and factor Va^{R506Q} in the test (see Results). Plasma samples were assayed within 1 h of thawing, although samples, kept at room temperature for 20 h, could be assayed without loss of assay accuracy. The intra-assay variation of the test was 3% (n = 17), the inter-assay variation is 4% (n = 20).

Semi-automated Method for the Detection of APC Resistance

Plasma samples were diluted 750 times in a 25 mM Hepes buffer (pH 7.5), containing 175 mM NaCl, 5 mM $CaCl_2$ and 5 mg/ml BSA, (buffer II). After prewarming two aliquots of 400 µl of the same plasma sample at 37° C, 16 µl of a mixture of 67 nM thrombin and 667 µM phospholipids (10/90 DOPS/DOPC M/M) without (sample A) or with 10 nM APC (sample B) was added yielding final concentrations of 4 nM thrombin, 20 µM phospholipid vesicles with or without 0.39 nM APC, respectively. After 20 min incubation, i.e. activation of plasma factor V (sample A) or activation and inactivation of plasma factor V (sample B) the reaction was stopped by the addition of 4500 µl cold buffer I (4° C). After dilution of the APC-treated plasma sample APC-catalyzed factor Va inactivation at 37° C is slowed down ~100-fold and becomes considerably slower than the rate of spontaneous loss of factor Va cofactor activity (<0.3 %/min at 37° C). Since spontaneous loss of factor Va activity occurs both in non-treated and APC-treated plasma samples any loss of factor Va activity during the preincubation/incubation procedure in the ACL-300 will not affect the APC-sr.

The cofactor activity of factor Va present in the plasma samples was determined on an ACL-300 Research Automated Coagulation apparatus (Instrumentation Laboratory, Milan, Italy). Prothrombin activation was started by mixing 50 µl aliquots of the plasma dilutions with 50 µl 2 µM prothrombin, 0.6 nM factor Xa and 40 µM phospholipids (10/90 DOPS/DOPC M/M) in 25 mM Hepes (pH 7.5), 175 mM NaCl. After 2 min prothrombin activation was stopped by mixing with 50 µl of 60 mM EDTA in 25 mM Hepes (pH 7.5), 175 mM NaCl, 0.5 mg/ml ovalbumin and 2.35 mM S2238 and the thrombin present was assayed by monitoring S2238 conversion for 1 min at 405 nm. Under the conditions described above S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample.

Statistics

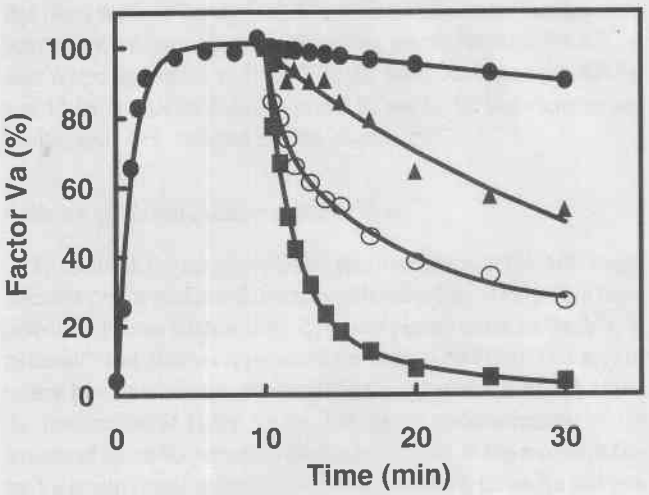
Data are presented as ranges of values observed, median values or as mean ± S.E.M. The Student's t-test was used to determine the significance of differences between test results of groups of plasmas.

Results

APC-mediated Inactivation of Factor Va Generated in Normal-pooled Plasma and in Plasmas from Heterozygous and Homozygous APC-resistant Individuals

The chromogenic method to screen plasmas for APC resistance is actually based on earlier publications from our group concerning a sensitive assay for factor Va (33), kinetic analysis of APC-catalyzed inactivation of factor Va and factor Va^{R506Q} (14) and the effects of protein S and factor Xa thereon (35).

Recently, we have shown (14) that APC inactivates normal factor Va 20 times faster than factor Va^{R506Q}. The difference between the observed rates of inactivation of factor Va and factor Va^{R506Q} was



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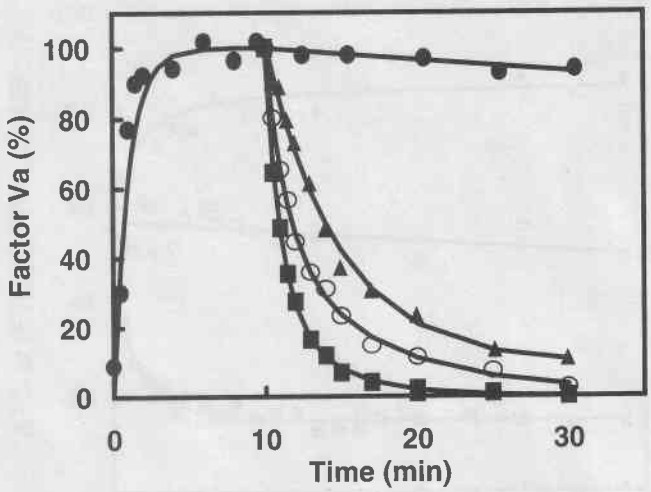

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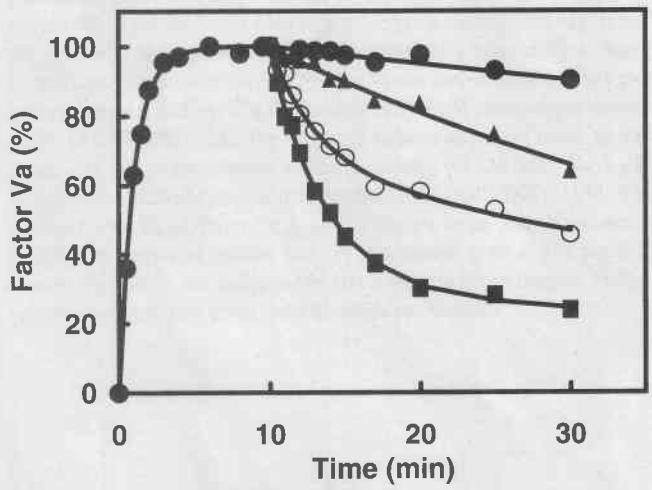

C

Fig. 1 Effect of APC on the activity of factor Va generated in normal plasma and in plasma from heterozygote and homozygote APC-resistant individuals. Normal-pooled plasma (■), plasma from heterozygous (○) or homozygous (▲) APC-resistant individual was diluted 1000 times in buffer I and factor V was activated at 37° C with 4 nM thrombin in the presence of phospholipid vesicles (10/90 DOPS/DOPC M/M). After 10 min APC with or without protein S was added to inactivate factor Va. The formation and loss of factor Va cofactor activity was followed as described in "Materials and Methods". For each plasma sample the factor Va activity is given as % of its plateau value. Time courses of factor V activation in the different plasmas and the spontaneous loss of factor Va activity were indistinguishable and are therefore given in the figure as a single averaged curve (●) for the three different plasmas. The final concentrations of components during the inactivation and assay procedures were: A) 0.2 nM APC and 21.7 μM phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture; B) 0.2 nM APC, 200 nM protein S and 21.7 μM phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture and C) 0.2 nM APC and 21.7 μM phospholipid vesicles during inactivation and 10 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture

maximal when the loss of cofactor activity of factor Va was monitored in prothrombin activation mixtures that contained a low factor Xa concentration (e.g. 0.3 nM, cf. ref. 14). Furthermore, we reported that differences in the rates of inactivation of factor Va and factor Va^{R506Q} were less pronounced or even nullified when factor Va was inactivated in the presence of protein S and/or factor Xa (35).

Since the plasma of normal healthy volunteers contains ~ 25 nM factor V (36) and since our factor Va assay allows quantitation of factor Va as low as 0.2 pM it is possible to determine the sensitivity of plasma factor Va towards APC at high plasma dilutions (>1000-fold), independent from and unaffected by other plasma components. The procedure that we have developed for distinguishing normal and APC-resistant plasmas basically consists of three steps: 1) activation of the factor V present in a highly diluted plasma sample with thrombin, 2) incubation of factor Va in the presence of phospholipids with or without APC and 3) quantification of factor Va cofactor activity. In order to obtain optimal differentiation between plasmas from normal healthy individuals, heterozygous and homozygous APC individuals the inactivation of factor Va should theoretically be performed in the absence of factor Xa and protein S (35) and the cofactor activity of factor Va should be assayed at a low factor Xa concentration (14).

Fig. 1A shows that under these conditions the factor Va formed by thrombin in a normal plasma sample was indeed readily inactivated by 0.2 nM APC whereas the factor Va present in the plasma sample from a heterozygous APC-resistant patient showed intermediate sensitivity and that in plasma from a homozygous APC-resistant patient was very slowly inactivated by APC. The differences in response towards APC were much less pronounced when protein S was present during factor Va inactivation (Fig. 1B) or when the loss of cofactor activity of factor Va was assessed in a prothrombinase mixture that contained a high concentration of factor Xa (Fig. 1C).

For practical reasons it is preferable that an APC-resistance test is based on measurement of sensitivity of plasma factor Va towards APC at a single time point. Quantitative comparison of the plasma samples was facilitated by expressing their response towards APC in terms of the so-called APC sensitivity ratio (APC-sr, cf. ref. 2, 11, 17-18). The APC-sr is defined as the ratio of the factor Va activities determined after incubation of the plasma factor Va in the presence and absence of APC and was multiplied by 100 to obtain integers (APC-sr = {factor Va_{+APC}/factor Va_{-APC}} × 100). It appeared that under the reaction conditions of the experiment presented in Fig. 1A, clear differences between the APC-sr of the various plasmas were obtained for incuba-

tion times between 2 and 15 min. Therefore, in the case of a single time point assay, we have arbitrarily chosen for determination of the APC-sr after 6 min incubation with APC. Under these conditions the APC-sr was 13 for normal pooled plasma and 50 and 81 for heterozygous and homozygous APC-resistant plasma, respectively.

Influence of Plasma Dilution on the APC-sr

To establish the range of plasma dilutions at which the APC resistance test can be performed, we have determined the APC-sr as a function of the plasma dilution (Fig. 2). At low plasma dilutions the APC-sr of the different plasmas approached the same value (~50). This may be caused by effects of other plasma proteins (e.g. protein S, cf. ref. 35) on the inactivation of factor Va by APC or by underestimation of the amount of factor Va present in the plasma sample. When too much factor Va is introduced in the factor Va assay mixture, factor Xa and prothrombin become limiting, which will cause an underestimation of the factor Va concentration especially in the plasma sample that was not treated with APC.

Reliable differences between the APC-sr of normal and APC-resistant plasmas were obtained when the test was performed on plasma samples that were diluted 500 to 5000 times. At higher dilutions of the plasma samples, the thrombin used to activate factor V in the plasma sample significantly contributed to the thrombin determined in the factor Va assay. Correction for this contribution (typically <5% in the absence of APC, and 5-25% in the presence of APC) required subtraction of an assay blank in which plasma was substituted with buffer.

Test Results for a Population of Normal Plasmas and Plasmas from Heterozygous or Homozygous APC-resistant Individuals

Plasmas from a number of normal (n = 33), heterozygous (n = 17) and homozygous individuals (n = 7), both men and women, were screened using the method described in the previous paragraphs. For all plasmas the presence or absence of the factor V^{R506Q} mutation was verified by genetic analysis of genomic DNA samples. In agreement with the results presented in previous paragraphs for the single plasmas, clear differences were observed for the three different groups of plasmas (Fig. 3). The APC-sr values of the plasmas actually divided into three populations: 12.3 ± 0.38 (mean ± SEM), for normal plasmas, 42.3 ± 1.0 for the plasmas of heterozygous APC-resistant individuals and 85.6 ± 0.63 for the plasmas of homozygous APC-resistant individuals. The ranges of APC-sr observed for these three populations were 8-20, 35-50 and 82-88 respectively. There was no overlap between the three groups of plasmas, i.e. the APC-sr of homozygous APC- resistant individuals was significantly higher than the APC-sr of heterozygous APC-resistant individuals ($p < 0.0001$). A similar difference was observed between the APC-sr of the plasma of heterozygous APC-resistant individuals and the plasma of normal individuals. The outcome of the chromogenic APC resistance test was in full agreement with the results obtained by DNA analysis.

The Chromogenic APC Resistance Test and Plasma with Coagulation Abnormalities

APC resistance tests that are based on measurement of the effect of APC on the APTT give less reliable results in plasmas with abnormal APTT values e. g. in the case of coagulation factor deficiencies (17, 20, 21), anticoagulant treatment with heparin or oral anticoagulants (2, 4, 6, 17, 23), the presence of Lupus anticoagulant (22), pregnancy (21) or the

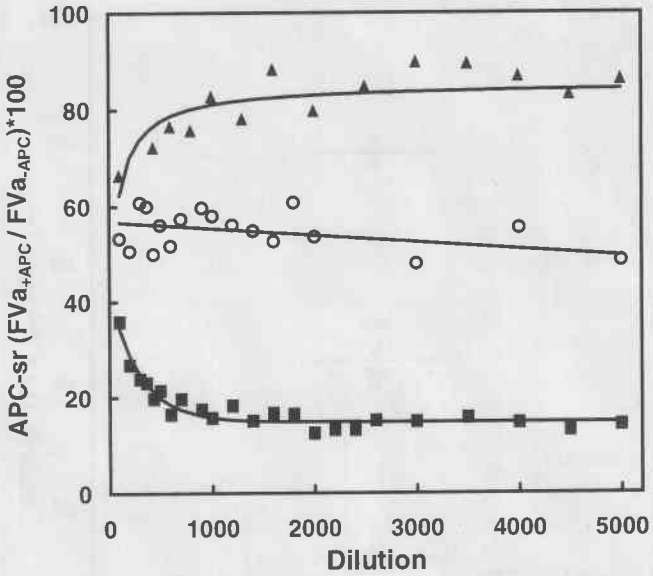

Fig. 2 The influence of the plasma dilution on the response of the various plasmas to APC. The factor V present in varying dilutions of normal-pooled plasma (■) or plasma from a heterozygous (○) or a homozygous APC-resistant individual (▲) was activated by incubating the plasma dilution during 10 min with 4 nM thrombin at 37° C. Factor Va was further incubated for 6 min with or without 0.2 nM APC in the presence of 21.7 μM phospholipid vesicles (10/90 DOPS/DOPC M/M). The remaining cofactor activity of factor Va was determined in a prothrombinase mixture containing 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles (10/90 DOPS/DOPC M/M). The APC-sr (factor Va_{+APC}/factor Va_{-APC}) was corrected for an assay blank, determined with a reaction mixture that did not contain plasma. For practical reasons, the APC-sr was multiplied by 100 in order to obtain integers. Further experimental details are given under Materials and Methods

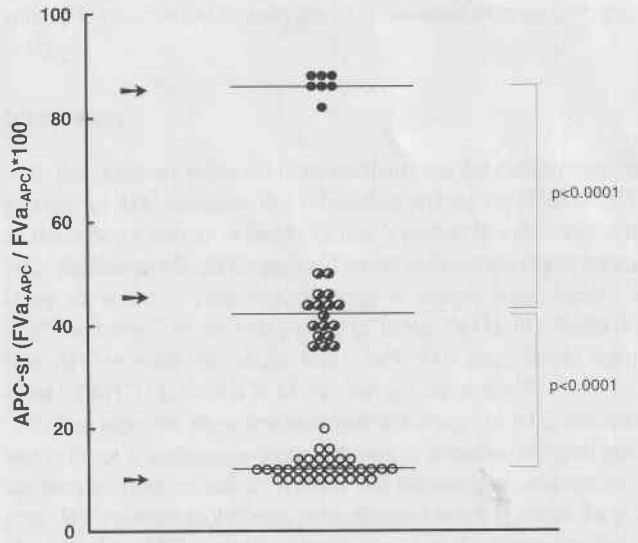

Fig. 3 Range of APC-sr of plasmas from healthy volunteers and heterozygous and homozygous APC-resistant individuals. The effect of APC on the cofactor activity of factor Va generated in a 1/1000 dilution of the plasma samples was determined as described in the Materials and Methods (cf. Fig. 2). The plasma samples were classified as belonging to normal healthy individuals (○), heterozygous APC-resistant individuals (◐) or homozygous APC-resistant individuals (●) by DNA analysis. The data obtained were plotted as APC-sr = factor Va_{+APC}/factor Va_{-APC} multiplied by 100. Median values are indicated per group. P-values are given. The position of the theoretical APC-sr (see Discussion) is indicated per group of plasma samples (→)

Table 1 APC-sr for normal plasmas and plasmas with abnormal coagulation properties

Plasma	APC-sr[#]	range	n
Plasma from normal individuals	12.3 ± 0.38	8-20	33
Heterozygous APC resistant plasma	41.9 ± 1.0	35-50	17
Homozygous APC resistant plasma	86.0 ± 0.63	82-88	7
Protein S deficient plasma*	12.8 ± 0.70	12-14	2
Plasma (oral anticoagulation)	15.4 ± 1.20	12-20	8
Plasma (Heparin treatment)	15.9 ± 0.74	14-18	4
Plasma (oral contraception)	13.1 ± 0.49	10-18	15
Plasma (pregnancy)	12.6 ± 0.61	12-14	3
Plasma (Lupus anticoagulans)	12.0 ± 0.96	10-14	3

[#] The APC-sr for the different plasmas was determined as described in the legend of Fig. 2. Mean ± S.E.M. are given, n: number of different plasma samples tested.

* immunodepleted and plasma from a heterozygous protein S deficient patient (<5% free protein S antigen)

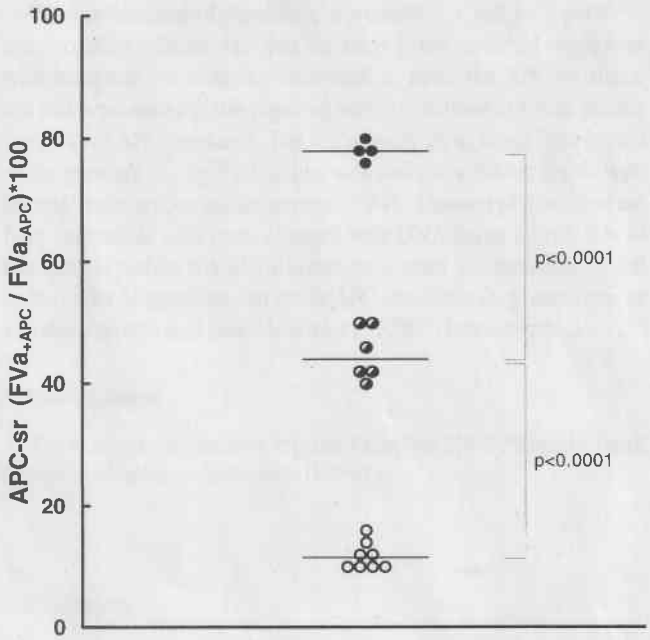

Fig. 4 Range of APC sensitivity ratios of plasmas from healthy volunteers and heterozygous and homozygous APC-resistant individuals determined by a semi-automated method. The APC-sr (factor Va_{+APC}/factor Va_{-APC}) × 100 of various plasmas was determined on an ACL-300 Research Automated Coagulation apparatus as described in the Materials and Methods. The plasma samples were classified as belonging to normal healthy individuals (○), heterozygous APC-resistant individuals (◕) or homozygous APC-resistant individuals (●) by DNA analysis. Median values are indicated per group

use of oral contraceptives (24, 25). Since our test is performed on highly diluted plasma samples it is only dependent on plasma factor V and is not dependent on other plasma components. Hence, it is not surprising that for all "abnormal plasmas" tested the APC-sr values obtained by our assay were within the range of normal plasmas (Table 1).

Modification of the Chromogenic APC Resistance Test for Use on an Autoanalyzer

Although the test described above is rather easy to perform we have adapted the assay for use on an ACL-300 Research Automated Coagulation Apparatus. In this application the plasma dilution, the activation of factor V and inactivation of factor Va were performed outside the apparatus but the factor Va was determined on the autoanalyzer. It appeared possible to activate plasma factor V and inactivate the factor Va with APC in a single step by incubating the plasma dilution with 20 μM phospholipids, 4 nM thrombin in the absence or presence of 0.4 nM APC during 20 min. After this incubation the sample was diluted 12-fold with cold Hepes buffer and stored on ice in order to block further factor Va inactivation. The factor Va present in the plasma samples thus obtained appeared to be stable for at least one hour, which allowed a factor Va determination on the autoanalyzer. In the autoanalyzer the treated plasma sample was mixed with a solution containing prothrombin, factor Xa and phospholipid vesicles (t = 0 min) and subsequently with a S2338/EDTA mixture (t = 2 min) to stop prothrombin activation and to determine thrombin. Under the chosen assay conditions the thrombin formed was proportional to the amount of factor Va present in the plasma sample. The results obtained with the semi-automated method are in good agreement with the data shown in Fig. 3 and also gave a distinct APC-sr for the normal plasmas and the heterozygous and homozygous APC-resistant plasmas. The differences between the three groups of plasmas tested were significant ($p < 0.0001$). The APC-sr (mean ± SEM) for the three groups of plasmas tested were 12.2 ± 0.24, 45.3 ± 0.72 and 77.3 ± 0.3 for the plasmas of normal, heterozygous APC-resistant and homozygous APC-resistant individuals respectively.

Discussion

In this paper we present a new functional test for the screening of plasmas for APC resistance that is based on probing the effect of APC on the cofactor activity of factor Va that is present in a thrombin-activated plasma sample. APC-catalyzed loss of cofactor activity of normal factor Va is due to proteolytic cleavage of peptide bonds located at Arg^{506} and Arg^{306} of the heavy chain of factor Va (12-14). Factor Va from APC-resistant individuals (factor Va^{R506Q}) is much slower inactivated by APC (14) since it lacks the cleavage site at Arg^{506}.

In this paper we show that maximal differences in APC-mediated loss of factor Va cofactor activities, present in thrombin-activated plasma samples from normal individuals and heterozygous and homozygous APC-resistant individuals, were obtained when 1) factor Va was inactivated by APC in a high plasma dilution in the absence of factor Xa and protein S and when 2) the factor Va cofactor activity was determined in a prothrombinase mixture containing a low factor Xa concentration (Fig. 1).

This led to a rapid and simple method for the screening of plasmas for APC resistance. Our procedure is based on comparison of the amounts of factor Va cofactor activity present in thrombin-activated plasma samples that were incubated during 6 min with phospholipid vesicles either in the absence or presence of 0.2 nM APC. The anticoagulant response of a given plasma sample to APC can be easily

quantitated from the so-called APC sensitivity ratio (APC-sr) which is defined as the ratio of amounts of factor Va present in the APC-treated and the non-treated plasma sample. The chosen reaction conditions allow full distinction between the normal plasmas and the plasmas from individuals that were shown to be heterozygous or homozygous APC resistant by DNA analysis (Fig. 3). This is in contrast to results obtained with APTT-based APC-resistance assays, in which borderline zones for the APC response are observed in which it is difficult to distinguish normal, heterozygous and homozygous APC-resistant individuals (11, 15, 37) unless the plasma sample is diluted in factor V-deficient plasma (38, 39) or buffer (40). The functional method for screening of APC resistance described in this paper gives results that are fully compatible with those obtained by DNA analysis. This is not surprising since the determination is performed on highly diluted plasma samples which makes the assay independent of other plasma components that may affect inactivation or quantitation of the factor Va present in the plasma sample. This has the additional advantage that our assay is not affected by conditions that may interfere with coagulation-based tests such as coagulation factor abnormalities (20, 21), the presence of lupus anticoagulants (22), anticoagulant treatment (2, 4, 6, 17, 23), pregnancy (21) or the use of oral contraceptives (24, 25) (Table 1). A number of the heterozygote and homozygote APC-resistant individuals had been suffering from episodes of venous thromboembolism. In the prothrombinase-based assay they were indistinguishable from healthy APC-resistant individuals.

It should be emphasized that the actual value of the APC-sr is dependent on reaction conditions such as pH, temperature, the concentrations of APC and phospholipid vesicles and the time of incubation with APC. When the reaction conditions are not strictly controlled, different APC-sr may be obtained. Day to day variation of the APC-sr can, however, be minimized by predetermining the APC-sr of a normal pooled plasma as a function of the incubation time with APC and taking in the actual test an incubation time that yields an APC-sr of 10-15 in the pilot experiment.

Actually, the APC-sr obtained in the experiment presented in Fig. 3 should equal a theoretical value that can be calculated using rate constants for APC-catalyzed cleavage of the peptide bonds located at Arg^{506} and Arg^{306} reported in an earlier paper from our group (14). Using rate constants of 6.7×10^{-5} s^{-1} for the spontaneous loss of factor Va cofactor activity and 4.3×10^{7} M^{-1} s^{-1} for APC-catalyzed cleavages at Arg^{506} and 2.3×10^{6} $M^{-1}s^{-1}$ for cleavage at Arg^{306} (14), theoretical APC-sr of 10, 46 and 85 are calculated for the plasma of normal individuals, heterozygous (assuming 50% normal factor V and 50% factor V^{R506Q}) and homozygous APC-resistant individuals, respectively. The theoretical values for the APC-sr closely approach the values experimentally obtained (Fig. 3).

We have shown that it is possible to use an autoanalyzer in order to increase the number of samples that can be tested simultaneously. In the semi-automated method the activation of plasma factor V by thrombin and the inactivation of factor Va by APC occur in a single step in a 37° C waterbath and the amount of factor Va present in the plasma sample is determined on an autoanalyzer by subsequently mixing the plasma sample with prothrombinase components (phospholipids, Ca^{2+} ions, prothrombin and factor Xa) and with a solution containing the thrombin-specific chromogenic substrate S2238 in EDTA (to block further prothrombin activation). When the reaction conditions and the incubation times were chosen properly, the rate of S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample and values for APC-sr allowed discrimination between normal, heterozygous and homozygous APC-resistant plasmas (Fig. 4).

The combination of reliability, reproducibility and easy performance, together with the fact that the assay is not disturbed in plasmas with abnormal coagulation characteristics, make the APC-resistance test that is presented in this paper an excellent method to screen plasma samples for APC resistance. The test actually gives direct information on the presence of Arg^{506}→Gln mutation and gives distinct results with normal, homozygous and heterozygous APC-resistant plasmas that are fully compatible with those obtained with DNA-based assays. It is to be expected that the test will also recognize other abnormalities related to the factor V gene that can cause APC resistance (e.g. mutations or variable expression of factor V alleles in R506Q heterozygotes).

Acknowledgements

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References

1. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: Prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 1993; 90: 1004-8.
2. Koster T, Rosendaal FR, De Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to a poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342: 1503-6.
3. Griffin JH, Evatt B, Wideman C, Fernandez JA. Anticoagulant protein C pathway defective in a majority of thrombophilic patients. Blood 1993; 82: 1989-93.
4. Cadroy Y, Sié P, Boneu B. Frequency of a defective response to activated protein C in patients with a history of venous thrombosis. Blood 1994; 83: 2008-9.
5. Svensson PJ, Dahlbäck B. Resistance to activated protein C as a basis for venous thrombosis. N Engl J Med 1994; 330: 517-22.
6. Halbmayer WM, Haushofer A, Schön R, Fischer M. The prevalence of poor anticoagulant response to activated protein C (APC Resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects. Blood Coagul Fibrinol 1995; 5: 51-7.
7. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, De Ronde H, Van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369: 64-7.
8. Voorberg J, Roelse J, Koopman R, Büller H, Berends F, Ten Cate JW, Mertens K, Van Mourik JA. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 1994; 343: 1535-6.
9. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet 1994; 343: 1361-62.
10. Alhenc-Gelas M, Gandrille S, Aubry M-L, Emmerich J, Fiessinger J-N, Aiach M. Unexplained thrombosis and factor V Leiden mutation. Lancet 1994; 344: 555-6.
11. Zöller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. J Clin Invest 1994; 94: 2521-4.
12. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem 1994; 269: 31869-80.
13. Kalafatis M, Bertina RM, Rand MR, Mann KG. Characterization of the molecular defect in factor V^{R506Q}. J Biol Chem 1995: 270: 4053-7.
14. Nicolaes GAF, Tans G, Thomassen MCLGD, Hemker HC, Pabinger I, Varadi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of

functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. J Biol Chem 1995; 270: 21158-66.
15. Dahlbäck B. Resistance to activated protein C, the Arg^{506} to Gln mutation in the factor V gene and venous thrombosis. Thromb Haemost 1995; 73: 739-42.
16. Gandrille S, Alhenc-Gelas M, Aiach M. A rapid screening method for the factor V Arg506—>Gln mutation. Blood Coagul Fibrinol 1995; 6: 245-8.
17. De Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thromb Haemost 1994; 72: 880-6.
18. Rosén S, Johansson K, Lindberg K, Dahlbäck B. Multicenter evaluation of a kit for activated protein C resistance on various coagulation instruments using plasmas from healthy individuals. Thromb Haemost 1994; 72: 255-60.
19. Nicolaes GAF, Thomassen MCLGD, Béguin S, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in normal and APC resistant individuals. Thromb Haemost, submitted for publication.
20. Kraus M, Zander N, Fickenscher K. Coagulation assay with improved specificity to factor V mutants insensitive to activated protein C. Thromb Res 1995; 80: 255-64.
21. Vasse M, Leduc O, Borg J-Y, Chrétien MH, Monconduit M. Resistance to activated protein C: evaluation of three functional assays. Thromb Res 1994; 76: 47-59.
22. Bokarewa MI, Bremme K, Falk G, Sten-Linder M, Egberg N, Blombäck M. Studies on phospholipid antibodies, APC-resistance and associated mutation in the coagulation factor V gene. Thromb Res 1995; 78: 193-200.
23. Cadroy Y, Sié P, Alhenc-Gelas M, Aiach M. Evaluation of APC resistance in the plasma of patients with Q506 mutation of factor V (factor V Leiden) and treated by oral anticoagulants. Thromb Haemost 1995; 73: 734-5.
24. Henkens CMA, Bom VJJ, Seinen AJ, Van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. Thromb Haemost 1995; 73: 402-4.
25. Olivieri O, Friso S, Manzato F, Guella A, Bernardi F, Lunghi B, Girelli D, Azzini M, Brocco G, Russo C, Corrocher R. Resistance to activated protein C in healthy women taking oral contraceptives. Br J Haematol 1995; 91: 465-70.
26. DiScipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and Protein S. Biochemistry 1977; 16: 698-706.
27. Bock PE, Craig PA, Olsen ST, Singh P. Isolation of human blood coagulation factor α-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active site titration with fluorescin mono-p-guanidinobenzoate. Arch Biochem Biophys 1989; 273: 375-88.
28. Rosing J, Bakker HM, Thomassen MCLGD, Hemker HC, Tans G. Characterization of two forms of human factor Va with different cofactor activities. J Biol Chem 1993; 268: 21130-6.
29. Böttcher CJF, van Gent CM, Pries C. A rapid and sensitive sub-micro phosphorus determination. Anal Chim Acta 1961; 24: 203-7.
30. Sala N, Owen WG, Collen D. A functional assay for protein C in human plasma. Blood 1984; 63: 671-5.
31. Rosing J, Tans G, Govers-Riemslag JWP, Zwaal RFA, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem 1980; 255: 274-83.
32. DiScipio RG, Davie EW. Characterization of protein S a gamma-carboxyglutamic acid containing protein from bovine and human plasma. Biochemistry 1979; 18: 899-904.
33. Tans G, Rosing J, Thomassen MCLGD, Heeb MJ, Zwaal RFA, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood 1991; 77: 2641-8.
34. Beauchamp NJ, Daly ME, Hampton KK, Cooper PC, Preston FE, Peake IR. High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. Br J Haematol 1994; 88: 219-22.
35. Rosing J, Hoekema L, Nicolaes GAF, Thomassen MCLGD, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. J Biol Chem 1995; 270: 27852-8.
36. Tracy PB, Eide LL, Bowie EJW, Mann KG. Radioimmunoassay of factor V in human plasma and platelets. Blood 1982; 60: 59-63.
37. Samaha M, Trossaert M, Conard J, Horellou MH, Elalamy I, Samama MM. Prevalence and patient profile in activated protein C resistance. Am J Clin Pathol 1995; 104:450-4.
38. Sun X, Evatt B, Griffin J. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. Blood 1994; 11: 3120-5.
39. Engel H, Zwang L, van Vliet HHDM, Michiels JJ, Stibbe J, Lindemans J. Phenotyping and genotyping of coagulation factor V Leiden. Thromb Haemost 1996; 75: 267-9.
40. Le DT, Donnely K, Sevilla B, Rapaport SI. Further experience with the TF-based FV assay for APC resistant FVa (APC-R). Blood 1995; 86: 202a.

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A Prothrombinase-based Assay for Detection of Resistance to Activated Protein C

Gerry A. F. Nicolaes[1], M. Christella L. G. D. Thomassen[1], Rene van Oerle[2], Karly Hamulyak[2], H. Coenraad Hemker[1], Guido Tans[1], Jan Rosing[1]

From the [1]Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM) and the [2]Department of Hematology, University Hospital, University of Limburg, Maastricht, The Netherlands

Summary

In this paper we present a new method for the detection of resistance to activated protein C (APC) that is based on direct measurement of the effect of APC on the cofactor activity of plasma factor Va. The factor V present in a diluted plasma sample was activated with thrombin and its sensitivity towards APC was subsequently determined by incubation with phospholipids and APC. The loss of factor Va cofactor activity was quantified in a prothrombinase system containing purified prothrombin, factor Xa and phospholipid vesicles and using a chromogenic assay for quantitation of thrombin formation. The reaction conditions were optimized in order to distinguish normal, heterozygous and homozygous APC-resistant plasmas. Maximal differences in the response of these plasmas towards APC were observed when factor Va was inactivated by APC in the absence of protein S and when the cofactor activity of factor Va was determined at a low factor Xa concentration (0.3 nM).

Addition of 0.2 nM APC and 20 μM phospholipid vesicles to a 1000-fold diluted sample of thrombin-activated normal plasma resulted in loss of more than 85% of the cofactor activity factor Va within 6 min. Under the same conditions, APC inactivated ~60% and ~20% of the factor Va present in plasma samples from APC-resistant individuals that were heterozygous or homozygous for the mutation $Arg^{506}\rightarrow Gln$ in factor V, respectively. Discrimination between the plasma samples from normal and heterozygous and homozygous APC-resistant individuals was facilitated by introduction of the so-called APC-sensitivity ratio (APC-sr). The APC-sr was defined as the ratio of the factor Va cofactor activities determined in thrombin-activated plasma samples after 6 min incubation with or without 0.2 nM APC and was multiplied by 100 to obtain integers (APC-sr = {factor Va_{+APC}/factor Va_{-APC}} × 100). Clear differences were observed between the APC-sr of plasmas from normal healthy volunteers (APC-sr: 8-20, n = 33) and from individuals that were heterozygous (APC-sr: 35-50, n = 17) or homozygous APC resistant (APC-sr: 82-88, n = 7). There was no mutual overlap between the APC-sr of normal plasmas and plasmas from heterozygous or homozygous APC resistant individuals ($p < 0.0001$). In all cases our test gave the same result as the DNA-based assay. Since the test is performed on a highly diluted plasma sample there is no interference by conditions that affect APC resistance tests that are based on clotting time determinations (e.g. coagulation factor deficiencies, oral anticoagulation, heparin treatment, the presence of lupus anticoagulants, pregnancy or the use of oral contraceptives). Furthermore, we show that part of the factor Va assay can be performed on an autoanalyzer which increases the number of plasma samples that can be handled simultaneously.

Correspondence to: Dr. J. Rosing, Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University of Limburg, PO Box 616, 6200 MD Maastricht, The Netherlands – FAX Number: +31 43 3670 988

Introduction

In 1993, Dahlbäck et al. (1) described a hereditary defect in the anticoagulant response to activated protein C (APC) that is associated with an increased risk for venous thrombosis. This abnormality, called APC resistance, was subsequently identified as the most common defect in thromboembolic patients (2-6) and was found in 20-50% of patients with venous thrombosis. APC resistance is associated with familial thrombophilia and is inherited as an autosomal dominant trait. The molecular defect responsible for APC resistance is a single point mutation in the gene encoding for coagulation factor V. This mutation results in the substitution of Arg^{506} by Gln in the heavy chain domain of factor V(a) (7-11). Since Arg^{506} constitutes one of three APC cleavage sites in factor Va (12), the mutated factor V(a), also described as factor V^{Leiden} or factor V^{R506Q}, is much less effectively inactivated by APC than normal factor Va (13, 14). Since APC-catalyzed inactivation of factor Va is a crucial step in the down-regulation of thrombin formation this likely explains the increased risk for venous thromboembolism in individuals with APC-resistant plasma.

The requirement for reliable detection of APC resistance became important when it was discovered that at least 2 to 4% of the Caucasian population is APC-resistant (2, 3, 6, 7). Several methods for the diagnosis of APC resistance have been described so far (15). Most of these methods are based on sequence analysis of genomic DNA (7, 16) or on measurement of the effect of APC on the activated partial thromboplastin time (APTT) (1, 17, 18) or on thrombin generation in plasma (19). APTT-based APC-resistance assays appear to be less reliable in the case of other coagulation disorders (17, 20-22), anticoagulant therapy (2, 4, 6, 23), pregnancy (21) or the use of oral contraceptives (24, 25).

In this paper, we report a different method for the identification of APC-resistant plasmas. The method is based on direct measurement of the effect of APC on the activity of factor Va in diluted thrombin-activated plasma samples and subsequent determination of factor Va cofactor activity in prothrombin activation.

Materials and Methods

Materials and Buffers

The phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. The chromogenic substrates D-Phe-

(pipecolyl)-Arg-pNA (S2238) and L-pyroGlu-Pro-Arg-pNA (S2366) were supplied by Chromogenix, Mölndal, Sweden. Recombinant human APC (rAPC) was a kind gift of Immuno AG, Vienna, Austria. Human protein S was purchased from Enzyme Research Laboratories, Swansea, United Kingdom. Human prothrombin and human factor X were purified as described by DiScipio et al. (26). Human factor X was converted into factor Xa by incubation with the purified factor X activator from Russel's viper venom and factor Xa was isolated from the activation mixture by chromatography on soybean trypsin inhibitor-Sepharose (27).

All proteins used in the APC resistance test were diluted in buffer I (25 mM Hepes [pH 7.5], 175 mM NaCl, 3 mM $CaCl_2$ and 5 mg/ml BSA).

Phospholipid Vesicle Preparations

Small unilamellar phospholipid vesicles composed of a mixture of DOPS/DOPC (10/90, M/M) were prepared as described earlier (28). Phospholipid concentrations were determined by phosphate analysis (29).

Protein Concentrations

APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al. (30). Thrombin concentrations were determined with the chromogenic substrate S2238 (31). Protein S concentrations were calculated from the A_{280} using an $A^{1\%}_{280}$ of 9.5 and Mr = 70,000 for protein S (32). Factor V and factor Va concentrations were determined by measuring their activity in prothrombin activation (14, 33) under conditions described in the legends to the figures.

Collection and Handling of Plasma Samples

Nine parts of blood from normal healthy volunteers or from individuals (both men and women) that were shown to be heterozygous or homozygous for the $Arg^{506}\rightarrow Gln$ mutation in factor V by DNA analysis (34) were collected in one part of 0.13 M trisodium citrate (pH 7.8). The blood was centrifuged twice for 15 minutes at 3,000 × g at room temperature. The platelet poor plasma thus obtained was stored at -80° C. The healthy volunteers had a normal APTT and prothrombin time, a normal response to APC and DNA analysis showed that they were not carrying the $Arg^{506}\rightarrow Gln$ mutation. A normal plasma pool was obtained by mixing the plasma of 84 different healthy volunteers (both men and women). Coagulation factor concentrations (including protein S and C) of all volunteers were within the normal range.

Chromogenic APC-resistance Test

The procedure that we have developed basically consists of three steps that are performed at 37° C: 1) activation of plasma factor V with thrombin, 2) incubation of factor Va with or without APC and 3) quantification of factor Va cofactor activity.

Step 1: To two tubes (samples A and B) with 215 µl of 1/1000 dilutions of the same plasma sample in buffer I, 5 µl of a mixture of 175 nM thrombin and 1 mM phospholipid vesicles (10/90 M/M DOPS/DOPC) were added to activate the factor V present in the plasma sample.

Step 2: After 10 min 10 µl buffer I was added to sample A and 10 µl purified rAPC (4.6 nM) was added to sample B and the incubation at 37° C was continued.

Step 3: After 6 min incubation with APC, 10 µl prothrombin (25 µM) and 10 µl factor Xa (7.5 nM) were added to the samples A and B in order to determine the factor Va cofactor activity present. After 1 min, the amount of thrombin generated in the reaction aliquots was quantitated by transferring a 10 µl aliquot into a disposable cuvette with 990 µl of a buffer containing 235 µM S2238 in 50 mM Tris (pH 7.5 at 37° C), 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA. The factor Va concentration in the plasma sample was calculated from the thrombin formed using a calibration curve made with known amounts of purified factor Va (34).

The final concentrations of essential reaction components in the different steps were: step 1) 1/1000 dilution of plasma sample, 4 nM thrombin and 22.7 µM DOPS/DOPC (10/90, M/M) vesicles; step 2), 0.2 nM APC and 21.7 µM DOPS/DOPC (10/90) vesicles; step 3) 0.25 µM prothrombin, 0.3 nM factor Xa and 20 µM DOPS/DOPC (10/90) vesicles.

The factor Va assay procedure (step 3) is essentially the same as reported earlier (33). However, in this particular application factor Va was assayed at 0.3 nM factor Xa since at this factor Xa concentration the residual cofactor activity of the reaction intermediate cleaved at Arg^{506} is negligible compared to native factor Va (14). This increased the difference in response between normal and factor Va^{R506Q} in the test (see Results). Plasma samples were assayed within 1 h of thawing, although samples, kept at room temperature for 20 h, could be assayed without loss of assay accuracy. The intra-assay variation of the test was 3% (n = 17), the inter-assay variation is 4% (n = 20).

Semi-automated Method for the Detection of APC Resistance

Plasma samples were diluted 750 times in a 25 mM Hepes buffer (pH 7.5), containing 175 mM NaCl, 5 mM $CaCl_2$ and 5 mg/ml BSA, (buffer II). After prewarming two aliquots of 400 µl of the same plasma sample at 37° C, 16 µl of a mixture of 67 nM thrombin and 667 µM phospholipids (10/90 DOPS/DOPC M/M) without (sample A) or with 10 nM APC (sample B) was added yielding final concentrations of 4 nM thrombin, 20 µM phospholipid vesicles with or without 0.39 nM APC, respectively. After 20 min incubation, i.e. activation of plasma factor V (sample A) or activation and inactivation of plasma factor V (sample B) the reaction was stopped by the addition of 4500 µl cold buffer I (4° C). After dilution of the APC-treated plasma sample APC-catalyzed factor Va inactivation at 37° C is slowed down ~100-fold and becomes considerably slower than the rate of spontaneous loss of factor Va cofactor activity (<0.3 %/min at 37° C). Since spontaneous loss of factor Va activity occurs both in non-treated and APC-treated plasma samples any loss of factor Va activity during the preincubation/incubation procedure in the ACL-300 will not affect the APC-sr.

The cofactor activity of factor Va present in the plasma samples was determined on an ACL-300 Research Automated Coagulation apparatus (Instrumentation Laboratory, Milan, Italy). Prothrombin activation was started by mixing 50 µl aliquots of the plasma dilutions with 50 µl 2 µM prothrombin, 0.6 nM factor Xa and 40 µM phospholipids (10/90 DOPS/DOPC M/M) in 25 mM Hepes (pH 7.5), 175 mM NaCl. After 2 min prothrombin activation was stopped by mixing with 50 µl of 60 mM EDTA in 25 mM Hepes (pH 7.5), 175 mM NaCl, 0.5 mg/ml ovalbumin and 2.35 mM S2238 and the thrombin present was assayed by monitoring S2238 conversion for 1 min at 405 nm. Under the conditions described above S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample.

Statistics

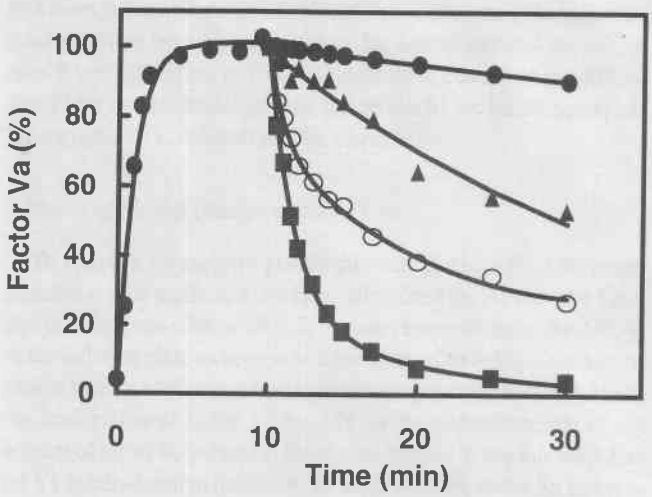
Data are presented as ranges of values observed, median values or as mean ± S.E.M. The Student's t-test was used to determine the significance of differences between test results of groups of plasmas.

Results

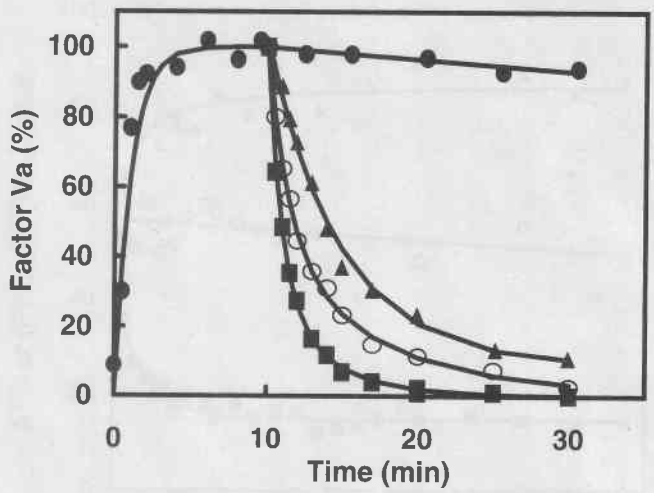
APC-mediated Inactivation of Factor Va Generated in Normal-pooled Plasma and in Plasmas from Heterozygous and Homozygous APC-resistant Individuals

The chromogenic method to screen plasmas for APC resistance is actually based on earlier publications from our group concerning a sensitive assay for factor Va (33), kinetic analysis of APC-catalyzed inactivation of factor Va and factor Va^{R506Q} (14) and the effects of protein S and factor Xa thereon (35).

Recently, we have shown (14) that APC inactivates normal factor Va 20 times faster than factor Va^{R506Q}. The difference between the observed rates of inactivation of factor Va and factor Va^{R506Q} was



A



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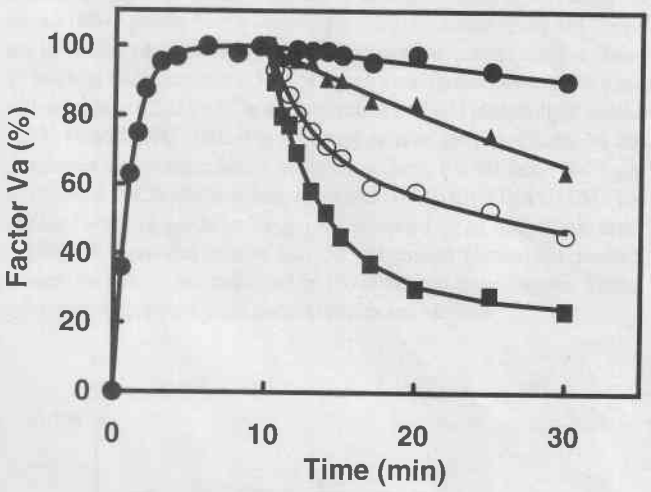

C

Fig. 1 Effect of APC on the activity of factor Va generated in normal plasma and in plasma from heterozygote and homozygote APC-resistant individuals. Normal-pooled plasma (■), plasma from heterozygous (○) or homozygous (▲) APC-resistant individual was diluted 1000 times in buffer I and factor V was activated at 37° C with 4 nM thrombin in the presence of phospholipid vesicles (10/90 DOPS/DOPC M/M). After 10 min APC with or without protein S was added to inactivate factor Va. The formation and loss of factor Va cofactor activity was followed as described in "Materials and Methods". For each plasma sample the factor Va activity is given as % of its plateau value. Time courses of factor V activation in the different plasmas and the spontaneous loss of factor Va activity were indistinguishable and are therefore given in the figure as a single averaged curve (●) for the three different plasmas. The final concentrations of components during the inactivation and assay procedures were: A) 0.2 nM APC and 21.7 μM phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture; B) 0.2 nM APC, 200 nM protein S and 21.7 μM phospholipid vesicles during inactivation and 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture and C) 0.2 nM APC and 21.7 μM phospholipid vesicles during inactivation and 10 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles in the factor Va assay mixture

maximal when the loss of cofactor activity of factor Va was monitored in prothrombin activation mixtures that contained a low factor Xa concentration (e.g. 0.3 nM, cf. ref. 14). Furthermore, we reported that differences in the rates of inactivation of factor Va and factor Va^{R506Q} were less pronounced or even nullified when factor Va was inactivated in the presence of protein S and/or factor Xa (35).

Since the plasma of normal healthy volunteers contains ~ 25 nM factor V (36) and since our factor Va assay allows quantitation of factor Va as low as 0.2 pM it is possible to determine the sensitivity of plasma factor Va towards APC at high plasma dilutions (>1000-fold), independent from and unaffected by other plasma components. The procedure that we have developed for distinguishing normal and APC-resistant plasmas basically consists of three steps: 1) activation of the factor V present in a highly diluted plasma sample with thrombin, 2) incubation of factor Va in the presence of phospholipids with or without APC and 3) quantification of factor Va cofactor activity. In order to obtain optimal differentiation between plasmas from normal healthy individuals, heterozygous and homozygous APC individuals the inactivation of factor Va should theoretically be performed in the absence of factor Xa and protein S (35) and the cofactor activity of factor Va should be assayed at a low factor Xa concentration (14).

Fig. 1A shows that under these conditions the factor Va formed by thrombin in a normal plasma sample was indeed readily inactivated by 0.2 nM APC whereas the factor Va present in the plasma sample from a heterozygous APC-resistant patient showed intermediate sensitivity and that in plasma from a homozygous APC-resistant patient was very slowly inactivated by APC. The differences in response towards APC were much less pronounced when protein S was present during factor Va inactivation (Fig. 1B) or when the loss of cofactor activity of factor Va was assessed in a prothrombinase mixture that contained a high concentration of factor Xa (Fig. 1C).

For practical reasons it is preferable that an APC-resistance test is based on measurement of sensitivity of plasma factor Va towards APC at a single time point. Quantitative comparison of the plasma samples was facilitated by expressing their response towards APC in terms of the so-called APC sensitivity ratio (APC-sr, cf. ref. 2, 11, 17-18). The APC-sr is defined as the ratio of the factor Va activities determined after incubation of the plasma factor Va in the presence and absence of APC and was multiplied by 100 to obtain integers (APC-sr = {factor Va_{+APC}/factor Va_{-APC}} × 100). It appeared that under the reaction conditions of the experiment presented in Fig. 1A, clear differences between the APC-sr of the various plasmas were obtained for incuba-

tion times between 2 and 15 min. Therefore, in the case of a single time point assay, we have arbitrarily chosen for determination of the APC-sr after 6 min incubation with APC. Under these conditions the APC-sr was 13 for normal pooled plasma and 50 and 81 for heterozygous and homozygous APC-resistant plasma, respectively.

Influence of Plasma Dilution on the APC-sr

To establish the range of plasma dilutions at which the APC resistance test can be performed, we have determined the APC-sr as a function of the plasma dilution (Fig. 2). At low plasma dilutions the APC-sr of the different plasmas approached the same value (~50). This may be caused by effects of other plasma proteins (e.g. protein S, cf. ref. 35) on the inactivation of factor Va by APC or by underestimation of the amount of factor Va present in the plasma sample. When too much factor Va is introduced in the factor Va assay mixture, factor Xa and prothrombin become limiting, which will cause an underestimation of the factor Va concentration especially in the plasma sample that was not treated with APC.

Reliable differences between the APC-sr of normal and APC-resistant plasmas were obtained when the test was performed on plasma samples that were diluted 500 to 5000 times. At higher dilutions of the plasma samples, the thrombin used to activate factor V in the plasma sample significantly contributed to the thrombin determined in the factor Va assay. Correction for this contribution (typically <5% in the absence of APC, and 5-25% in the presence of APC) required subtraction of an assay blank in which plasma was substituted with buffer.

Test Results for a Population of Normal Plasmas and Plasmas from Heterozygous or Homozygous APC-resistant Individuals

Plasmas from a number of normal (n = 33), heterozygous (n = 17) and homozygous individuals (n = 7), both men and women, were screened using the method described in the previous paragraphs. For all plasmas the presence or absence of the factor V^{R506Q} mutation was verified by genetic analysis of genomic DNA samples. In agreement with the results presented in previous paragraphs for the single plasmas, clear differences were observed for the three different groups of plasmas (Fig. 3). The APC-sr values of the plasmas actually divided into three populations: 12.3 ± 0.38 (mean ± SEM), for normal plasmas, 42.3 ± 1.0 for the plasmas of heterozygous APC-resistant individuals and 85.6 ± 0.63 for the plasmas of homozygous APC-resistant individuals. The ranges of APC-sr observed for these three populations were 8-20, 35-50 and 82-88 respectively. There was no overlap between the three groups of plasmas, i.e. the APC-sr of homozygous APC- resistant individuals was significantly higher than the APC-sr of heterozygous APC-resistant individuals ($p < 0.0001$). A similar difference was observed between the APC-sr of the plasma of heterozygous APC-resistant individuals and the plasma of normal individuals. The outcome of the chromogenic APC resistance test was in full agreement with the results obtained by DNA analysis.

The Chromogenic APC Resistance Test and Plasma with Coagulation Abnormalities

APC resistance tests that are based on measurement of the effect of APC on the APTT give less reliable results in plasmas with abnormal APTT values e. g. in the case of coagulation factor deficiencies (17, 20, 21), anticoagulant treatment with heparin or oral anticoagulants (2, 4, 6, 17, 23), the presence of Lupus anticoagulant (22), pregnancy (21) or the

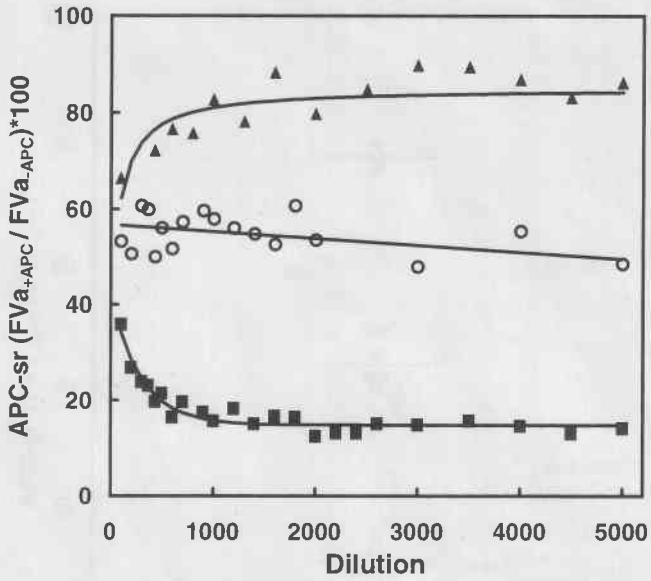

Fig. 2 The influence of the plasma dilution on the response of the various plasmas to APC. The factor V present in varying dilutions of normal-pooled plasma (■) or plasma from a heterozygous (○) or a homozygous APC-resistant individual (▲) was activated by incubating the plasma dilution during 10 min with 4 nM thrombin at 37° C. Factor Va was further incubated for 6 min with or without 0.2 nM APC in the presence of 21.7 μM phospholipid vesicles (10/90 DOPS/DOPC M/M). The remaining cofactor activity of factor Va was determined in a prothrombinase mixture containing 0.3 nM factor Xa, 1 μM prothrombin and 20 μM phospholipid vesicles (10/90 DOPS/DOPC M/M). The APC-sr (factor Va_{+APC}/factor Va_{-APC}) was corrected for an assay blank, determined with a reaction mixture that did not contain plasma. For practical reasons, the APC-sr was multiplied by 100 in order to obtain integers. Further experimental details are given under Materials and Methods

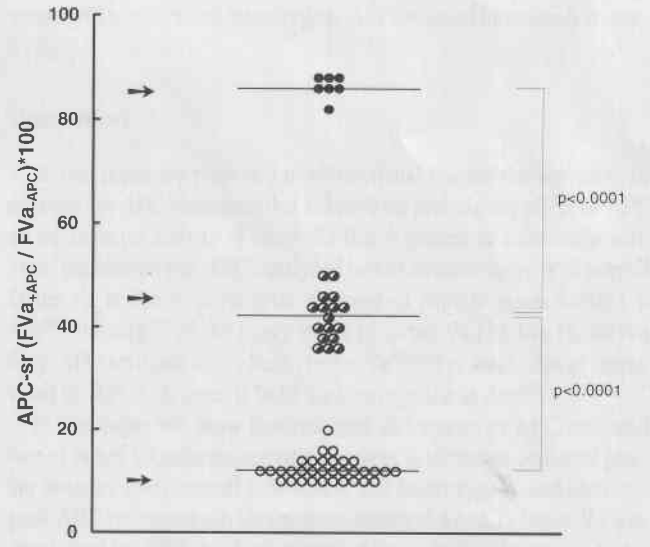

Fig. 3 Range of APC-sr of plasmas from healthy volunteers and heterozygous and homozygous APC-resistant individuals. The effect of APC on the cofactor activity of factor Va generated in a 1/1000 dilution of the plasma samples was determined as described in the Materials and Methods (cf. Fig. 2). The plasma samples were classified as belonging to normal healthy individuals (○), heterozygous APC-resistant individuals (◕) or homozygous APC-resistant individuals (●) by DNA analysis. The data obtained were plotted as APC-sr = factor Va_{+APC}/factor Va_{-APC} multiplied by 100. Median values are indicated per group. P-values are given. The position of the theoretical APC-sr (see Discussion) is indicated per group of plasma samples (→)

Table 1 APC-sr for normal plasmas and plasmas with abnormal coagulation properties

Plasma	APC-sr[#]	range	n
Plasma from normal individuals	12.3 ± 0.38	8-20	33
Heterozygous APC resistant plasma	41.9 ± 1.0	35-50	17
Homozygous APC resistant plasma	86.0 ± 0.63	82-88	7
Protein S deficient plasma*	12.8 ± 0.70	12-14	2
Plasma (oral anticoagulation)	15.4 ± 1.20	12-20	8
Plasma (Heparin treatment)	15.9 ± 0.74	14-18	4
Plasma (oral contraception)	13.1 ± 0.49	10-18	15
Plasma (pregnancy)	12.6 ± 0.61	12-14	3
Plasma (Lupus anticoagulans)	12.0 ± 0.96	10-14	3

[#]The APC-sr for the different plasmas was determined as described in the legend of Fig. 2. Mean ± S.E.M. are given, n: number of different plasma samples tested.

* immunodepleted and plasma from a heterozygous protein S deficient patient (<5% free protein S antigen)

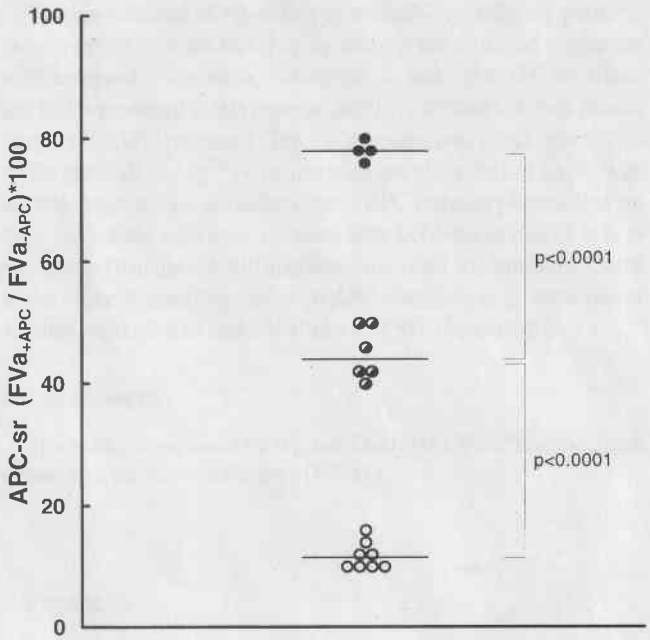

Fig. 4 Range of APC sensitivity ratios of plasmas from healthy volunteers and heterozygous and homozygous APC-resistant individuals determined by a semi-automated method. The APC-sr (factor Va_{+APC}/factor Va_{-APC}) × 100 of various plasmas was determined on an ACL-300 Research Automated Coagulation apparatus as described in the Materials and Methods. The plasma samples were classified as belonging to normal healthy individuals (○), heterozygous APC-resistant individuals (◕) or homozygous APC-resistant individuals (●) by DNA analysis. Median values are indicated per group

use of oral contraceptives (24, 25). Since our test is performed on highly diluted plasma samples it is only dependent on plasma factor V and is not dependent on other plasma components. Hence, it is not surprising that for all "abnormal plasmas" tested the APC-sr values obtained by our assay were within the range of normal plasmas (Table 1).

Modification of the Chromogenic APC Resistance Test for Use on an Autoanalyzer

Although the test described above is rather easy to perform we have adapted the assay for use on an ACL-300 Research Automated Coagulation Apparatus. In this application the plasma dilution, the activation of factor V and inactivation of factor Va were performed outside the apparatus but the factor Va was determined on the autoanalyzer. It appeared possible to activate plasma factor V and inactivate the factor Va with APC in a single step by incubating the plasma dilution with 20 μM phospholipids, 4 nM thrombin in the absence or presence of 0.4 nM APC during 20 min. After this incubation the sample was diluted 12-fold with cold Hepes buffer and stored on ice in order to block further factor Va inactivation. The factor Va present in the plasma samples thus obtained appeared to be stable for at least one hour, which allowed a factor Va determination on the autoanalyzer. In the autoanalyzer the treated plasma sample was mixed with a solution containing prothrombin, factor Xa and phospholipid vesicles (t = 0 min) and subsequently with a S2338/EDTA mixture (t = 2 min) to stop prothrombin activation and to determine thrombin. Under the chosen assay conditions the thrombin formed was proportional to the amount of factor Va present in the plasma sample. The results obtained with the semi-automated method are in good agreement with the data shown in Fig. 3 and also gave a distinct APC-sr for the normal plasmas and the heterozygous and homozygous APC-resistant plasmas. The differences between the three groups of plasmas tested were significant ($p < 0.0001$). The APC-sr (mean ± SEM) for the three groups of plasmas tested were 12.2 ± 0.24, 45.3 ± 0.72 and 77.3 ± 0.3 for the plasmas of normal, heterozygous APC-resistant and homozygous APC-resistant individuals respectively.

Discussion

In this paper we present a new functional test for the screening of plasmas for APC resistance that is based on probing the effect of APC on the cofactor activity of factor Va that is present in a thrombin-activated plasma sample. APC-catalyzed loss of cofactor activity of normal factor Va is due to proteolytic cleavage of peptide bonds located at Arg^{506} and Arg^{306} of the heavy chain of factor Va (12-14). Factor Va from APC-resistant individuals (factor Va^{R506Q}) is much slower inactivated by APC (14) since it lacks the cleavage site at Arg^{506}.

In this paper we show that maximal differences in APC-mediated loss of factor Va cofactor activities, present in thrombin-activated plasma samples from normal individuals and heterozygous and homozygous APC-resistant individuals, were obtained when 1) factor Va was inactivated by APC in a high plasma dilution in the absence of factor Xa and protein S and when 2) the factor Va cofactor activity was determined in a prothrombinase mixture containing a low factor Xa concentration (Fig. 1).

This led to a rapid and simple method for the screening of plasmas for APC resistance. Our procedure is based on comparison of the amounts of factor Va cofactor activity present in thrombin-activated plasma samples that were incubated during 6 min with phospholipid vesicles either in the absence or presence of 0.2 nM APC. The anticoagulant response of a given plasma sample to APC can be easily

quantitated from the so-called APC sensitivity ratio (APC-sr) which is defined as the ratio of amounts of factor Va present in the APC-treated and the non-treated plasma sample. The chosen reaction conditions allow full distinction between the normal plasmas and the plasmas from individuals that were shown to be heterozygous or homozygous APC resistant by DNA analysis (Fig. 3). This is in contrast to results obtained with APTT-based APC-resistance assays, in which borderline zones for the APC response are observed in which it is difficult to distinguish normal, heterozygous and homozygous APC-resistant individuals (11, 15, 37) unless the plasma sample is diluted in factor V-deficient plasma (38, 39) or buffer (40). The functional method for screening of APC resistance described in this paper gives results that are fully compatible with those obtained by DNA analysis. This is not surprising since the determination is performed on highly diluted plasma samples which makes the assay independent of other plasma components that may affect inactivation or quantitation of the factor Va present in the plasma sample. This has the additional advantage that our assay is not affected by conditions that may interfere with coagulation-based tests such as coagulation factor abnormalities (20, 21), the presence of lupus anticoagulants (22), anticoagulant treatment (2, 4, 6, 17, 23), pregnancy (21) or the use of oral contraceptives (24, 25) (Table 1). A number of the heterozygote and homozygote APC-resistant individuals had been suffering from episodes of venous thromboembolism. In the prothrombinase-based assay they were indistinguishable from healthy APC-resistant individuals.

It should be emphasized that the actual value of the APC-sr is dependent on reaction conditions such as pH, temperature, the concentrations of APC and phospholipid vesicles and the time of incubation with APC. When the reaction conditions are not strictly controlled, different APC-sr may be obtained. Day to day variation of the APC-sr can, however, be minimized by predetermining the APC-sr of a normal pooled plasma as a function of the incubation time with APC and taking in the actual test an incubation time that yields an APC-sr of 10-15 in the pilot experiment.

Actually, the APC-sr obtained in the experiment presented in Fig. 3 should equal a theoretical value that can be calculated using rate constants for APC-catalyzed cleavage of the peptide bonds located at Arg^{506} and Arg^{306} reported in an earlier paper from our group (14). Using rate constants of 6.7×10^{-5} s^{-1} for the spontaneous loss of factor Va cofactor activity and 4.3×10^{7} M^{-1} s^{-1} for APC-catalyzed cleavages at Arg^{506} and 2.3×10^{6} $M^{-1}s^{-1}$ for cleavage at Arg^{306} (14), theoretical APC-sr of 10, 46 and 85 are calculated for the plasma of normal individuals, heterozygous (assuming 50% normal factor V and 50% factor V^{R506Q}) and homozygous APC-resistant individuals, respectively. The theoretical values for the APC-sr closely approach the values experimentally obtained (Fig. 3).

We have shown that it is possible to use an autoanalyzer in order to increase the number of samples that can be tested simultaneously. In the semi-automated method the activation of plasma factor V by thrombin and the inactivation of factor Va by APC occur in a single step in a 37° C waterbath and the amount of factor Va present in the plasma sample is determined on an autoanalyzer by subsequently mixing the plasma sample with prothrombinase components (phospholipids, Ca^{2+} ions, prothrombin and factor Xa) and with a solution containing the thrombin-specific chromogenic substrate S2238 in EDTA (to block further prothrombin activation). When the reaction conditions and the incubation times were chosen properly, the rate of S2238 conversion appeared to be proportional with the amount of factor Va present in the plasma sample and values for APC-sr allowed discrimination between normal, heterozygous and homozygous APC-resistant plasmas (Fig. 4).

The combination of reliability, reproducibility and easy performance, together with the fact that the assay is not disturbed in plasmas with abnormal coagulation characteristics, make the APC-resistance test that is presented in this paper an excellent method to screen plasma samples for APC resistance. The test actually gives direct information on the presence of $Arg^{506} \rightarrow Gln$ mutation and gives distinct results with normal, homozygous and heterozygous APC-resistant plasmas that are fully compatible with those obtained with DNA-based assays. It is to be expected that the test will also recognize other abnormalities related to the factor V gene that can cause APC resistance (e.g. mutations or variable expression of factor V alleles in R506Q heterozygotes).

Acknowledgements

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References

1. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: Prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 1993; 90: 1004-8.
2. Koster T, Rosendaal FR, De Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to a poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 1993; 342: 1503-6.
3. Griffin JH, Evatt B, Wideman C, Fernandez JA. Anticoagulant protein C pathway defective in a majority of thrombophilic patients. Blood 1993; 82: 1989-93.
4. Cadroy Y, Sié P, Boneu B. Frequency of a defective response to activated protein C in patients with a history of venous thrombosis. Blood 1994; 83: 2008-9.
5. Svensson PJ, Dahlbäck B. Resistance to activated protein C as a basis for venous thrombosis. N Engl J Med 1994; 330: 517-22.
6. Halbmayer WM, Haushofer A, Schön R, Fischer M. The prevalence of poor anticoagulant response to activated protein C (APC Resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects. Blood Coagul Fibrinol 1995; 5: 51-7.
7. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, De Ronde H, Van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369: 64-7.
8. Voorberg J, Roelse J, Koopman R, Büller H, Berends F, Ten Cate JW, Mertens K, Van Mourik JA. Association of idiopathic venous thromboembolism with single point-mutation at Arg506 of factor V. Lancet 1994; 343: 1535-6.
9. Greengard JS, Sun X, Xu X, Fernandez JA, Griffin JH, Evatt B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. Lancet 1994; 343: 1361-62.
10. Alhenc-Gelas M, Gandrille S, Aubry M-L, Emmerich J, Fiessinger J-N, Aiach M. Unexplained thrombosis and factor V Leiden mutation. Lancet 1994; 344: 555-6.
11. Zöller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. J Clin Invest 1994; 94: 2521-4.
12. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem 1994; 269: 31869-80.
13. Kalafatis M, Bertina RM, Rand MR, Mann KG. Characterization of the molecular defect in factor V^{R506Q}. J Biol Chem 1995: 270: 4053-7.
14. Nicolaes GAF, Tans G, Thomassen MCLGD, Hemker HC, Pabinger I, Varadi K, Schwarz HP, Rosing J. Peptide bond cleavages and loss of

functional activity during inactivation of factor Va and factor Va^{R506Q} by activated protein C. J Biol Chem 1995; 270: 21158-66.
15. Dahlbäck B. Resistance to activated protein C, the Arg^{506} to Gln mutation in the factor V gene and venous thrombosis. Thromb Haemost 1995; 73: 739-42.
16. Gandrille S, Alhenc-Gelas M, Aiach M. A rapid screening method for the factor V Arg506—>Gln mutation. Blood Coagul Fibrinol 1995; 6: 245-8.
17. De Ronde H, Bertina RM. Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thromb Haemost 1994; 72: 880-6.
18. Rosén S, Johansson K, Lindberg K, Dahlbäck B. Multicenter evaluation of a kit for activated protein C resistance on various coagulation instruments using plasmas from healthy individuals. Thromb Haemost 1994; 72: 255-60.
19. Nicolaes GAF, Thomassen MCLGD, Béguin S, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in normal and APC resistant individuals. Thromb Haemost, submitted for publication.
20. Kraus M, Zander N, Fickenscher K. Coagulation assay with improved specificity to factor V mutants insensitive to activated protein C. Thromb Res 1995; 80: 255-64.
21. Vasse M, Leduc O, Borg J-Y, Chrétien MH, Monconduit M. Resistance to activated protein C: evaluation of three functional assays. Thromb Res 1994; 76: 47-59.
22. Bokarewa MI, Bremme K, Falk G, Sten-Linder M, Egberg N, Blombäck M. Studies on phospholipid antibodies, APC-resistance and associated mutation in the coagulation factor V gene. Thromb Res 1995; 78: 193-200.
23. Cadroy Y, Sié P, Alhenc-Gelas M, Aiach M. Evaluation of APC resistance in the plasma of patients with Q506 mutation of factor V (factor V Leiden) and treated by oral anticoagulants. Thromb Haemost 1995; 73: 734-5.
24. Henkens CMA, Bom VJJ, Seinen AJ, Van der Meer J. Sensitivity to activated protein C; influence of oral contraceptives and sex. Thromb Haemost 1995; 73: 402-4.
25. Olivieri O, Friso S, Manzato F, Guella A, Bernardi F, Lunghi B, Girelli D, Azzini M, Brocco G, Russo C, Corrocher R. Resistance to activated protein C in healthy women taking oral contraceptives. Br J Haematol 1995; 91: 465-70.
26. DiScipio RG, Hermodson MA, Yates SG, Davie EW. A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and Protein S. Biochemistry 1977; 16: 698-706.
27. Bock PE, Craig PA, Olsen ST, Singh P. Isolation of human blood coagulation factor α-factor Xa by soybean trypsin inhibitor-sepharose chromatography and its active site titration with fluorescin mono-p-guanidinobenzoate. Arch Biochem Biophys 1989; 273: 375-88.
28. Rosing J, Bakker HM, Thomassen MCLGD, Hemker HC, Tans G. Characterization of two forms of human factor Va with different cofactor activities. J Biol Chem 1993; 268: 21130-6.
29. Böttcher CJF, van Gent CM, Pries C. A rapid and sensitive sub-micro phosphorus determination. Anal Chim Acta 1961; 24: 203-7.
30. Sala N, Owen WG, Collen D. A functional assay for protein C in human plasma. Blood 1984; 63: 671-5.
31. Rosing J, Tans G, Govers-Riemslag JWP, Zwaal RFA, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem 1980; 255: 274-83.
32. DiScipio RG, Davie EW. Characterization of protein S a gamma-carboxyglutamic acid containing protein from bovine and human plasma. Biochemistry 1979; 18: 899-904.
33. Tans G, Rosing J, Thomassen MCLGD, Heeb MJ, Zwaal RFA, Griffin JH. Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. Blood 1991; 77: 2641-8.
34. Beauchamp NJ, Daly ME, Hampton KK, Cooper PC, Preston FE, Peake IR. High prevalence of a mutation in the factor V gene within the U.K. population: relationship to activated protein C resistance and familial thrombosis. Br J Haematol 1994; 88: 219-22.
35. Rosing J, Hoekema L, Nicolaes GAF, Thomassen MCLGD, Hemker HC, Varadi K, Schwarz HP, Tans G. Effects of protein S and factor Xa on peptide bond cleavages during inactivation of factor Va and factor Va^{R506Q} by activated protein C. J Biol Chem 1995; 270: 27852-8.
36. Tracy PB, Eide LL, Bowie EJW, Mann KG. Radioimmunoassay of factor V in human plasma and platelets. Blood 1982; 60: 59-63.
37. Samaha M, Trossaert M, Conard J, Horellou MH, Elalamy I, Samama MM. Prevalence and patient profile in activated protein C resistance. Am J Clin Pathol 1995; 104:450-4.
38. Sun X, Evatt B, Griffin J. Blood coagulation factor Va abnormality associated with resistance to activated protein C in venous thrombophilia. Blood 1994; 11: 3120-5.
39. Engel H, Zwang L, van Vliet HHDM, Michiels JJ, Stibbe J, Lindemans J. Phenotyping and genotyping of coagulation factor V Leiden. Thromb Haemost 1996; 75: 267-9.
40. Le DT, Donnely K, Sevilla B, Rapaport SI. Further experience with the TF-based FV assay for APC resistant FVa (APC-R). Blood 1995; 86: 202a.

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